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The objective of this contract research was to gain a detailed understanding of the molecular events involved in flavivirus RNA replication, with the ultimate aim being the identification of inhibitors of the process. We established yellow fever virus (YFV) as our study system and assembled all the necessary virologic, molecular biologic, and immunologic reagents for the detailed study of viral RNA replication. Methodologies for detection, identification, and analysis of YFV polypeptides and RNA species were in place. Several YFV protein coding regions were engineered into various surrogate expression systems, and the recombinant proteins thereby produced would have served as important sources of material for functional biochemical studies if not for the premature termination of this contract by the government. An RNA-dependent RNA polymerase (RDRP) activity derived from virus-infected cells was identified and extensively characterized. A specific, reproducible, and quantifiable assay for this activity was established. A number of peptide-based compounds were designed, synthesized, and tested for inhibition of the RDRP activity in this assay. Preliminary data suggested several were inhibitory. Molecular modeling of peptides was investigated for its utility in assisting in next generation inhibitor design.

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Introduction

The general objective of this contract research was to obtain a detailed understanding of the molecular processes involved in flavivirus RNA replication. Toward this goal, studies involving the isolation and dissection of the replication apparatus found in virus-infected cells, and work dealing with the production and isolation of individual flaviviral proteins and their use in reconstructing component replication activities, were pursued. It was hoped data from these *in vitro* studies would not only significantly contribute to the elucidation of mechanisms involved in flavivirus RNA replication, but would also allow development of important *in vitro* assays for the component activities making up the viral replicase function. Such assays would have then allowed pursuit of our ultimate goal: identification and evaluation of compounds capable of interfering with these *in vitro* replication activities.

This report summarizes work performed over the entire life of this contract. It should be duly noted that this contract was cancelled by the government prematurely. This report reflects our efforts over 21 months of what was originally a 60 month program. Consequently, many aspects of our work plan are incomplete.

Experimental System Set-up and Reagents

Since this final report represents the first third of the originally outlined complete research plan, much of the effort over the abbreviated term of this contract involved the establishment of the experimental model system and the procurement and generation of necessary reagents. These set-up activities are summarized below.

Virologic reagents.

We obtained yellow fever virus (YFV), strain 17D, from American Type Culture Collection (ATCC) and from C. Rice (Washington University). The virus received from Dr. Rice was generated from the transfection of an infectious RNA copy of the molecularly cloned virus (YFiv 5.2; ref. 1). This latter virus was propagated in SW-13 cells (obtained from ATCC). Conditions for virus plaquing were established, and seed virus stocks and high titer working stocks were prepared. Additional cell lines obtained for this project included Vero, C6/36 cells (both obtained from USAMRIID), and BHK-21 cells (obtained from ATCC).

Molecular biologic reagents.

A series of plasmids possessing YFV 17D cDNA inserts which collectively encompass the entire virus genome were graciously provided by C. Rice (clones p28III, pYFM 3.5, p34III, and pYF5'-3'ΔNP.9). Stocks of these plasmids were prepared.

Immunologic reagents.

Mouse hyperimmune ascites fluid to YFV was obtained from ATCC and from J. Dalrymple (USAMRIID). Small aliquots of a series of rabbit antisera specific to protein coding regions along the YFV genome (generated against *E. coli*-expressed YFV polypeptides) were provided by C. Rice. All of these reagents were evaluated in radioimmunoprecipitation (RIP) analyses of YFV-infected BHK and SW-13 cell lysates. They allowed the clear identification of YFV proteins prM, E, NS1, NS2A, NS2B, NS3, NS4B, and NS5. Viral proteins not immunoprecipitated with these reagents from cell lysates were C, M, and NS4A (NS4A has not yet been identified in YFV-infected cells). Evaluation of 25 JEV monoclonal antibodies (provided by J. Dalrymple) by RIP assay of YFV-infected cell lysates identified two antibodies (11-H12-1 and 12-F9-3) cross-reactive with the YFV E protein. Six (6) previously developed YFV-specific monoclonal antibodies [obtained from Dr. J. Schlesinger (University of Rochester) with the help of Dr. T. Monath (USAMRIID)] were tested for YFV protein-specific reactivities. Two of the monoclones were strongly reactive with NS1; four with the E protein.

We invested considerable effort in generating our own sequence-specific antibody reagents to YFV proteins. Two types of immunogens were prepared. First, a number of peptides (19 to 26mers) were synthesized representing sequences from the C, M, E, NS2A, NS2B, NS4A, NS4B, and NS5 protein coding regions (Fig. 1B). These synthetic peptides were chemically coupled (via MBS) to KLH, and the resulting conjugates were used to immunize rabbits. We also produced sequence-specific immunogens by expressing select regions of the YFV open reading frame (ORF) in an *E. coli* expression system. Our system employs the tac promoter and results in production of a fusion protein in which the first 23 amino acids of the resultant protein represent the cro protein of bacteriophage λ (2). The regions of the ORF so engineered are schematically depicted in Figure 1C. With one exception, bacteria harboring these plasmid constructs each produced high levels (10-50 mg/l) of the expected polypeptide. The exception was croNS2A; while plasmid constructs could be verified correct, transformed *E. coli* failed to produce YFV-specific protein from this region. The successfully expressed bacterial YFV fusion polypeptides were partially purified and used to immunize rabbits as previously described (3,4).

Antisera were evaluated for their ability to immuno-precipitate radiolabeled antigens from YFV-infected SW-13 cell lysates. Infected cells were labeled with ^{35}S-methionine (TRANS-^{35}S-Label, ICN), and cleared cell lysates were prepared by either an SDS denaturation method (2) or by a RIPA buffer procedure (5). Following standard immunoprecipitation protocols (2,3), aliquots of the immunoprecipitates were analyzed by SDS polyacrylamide gel electrophoresis, and the radiolabeled antigens were detected by fluorography. Figure 1 summarizes the results of our evaluation of the various antibody reagents. Of eleven synthetic peptide immunogens, five elicited useful antisera reactive with specific

YFV polypeptides (Fig. 1B). Each of the four *E. coli*-produced immunogens yielded positive antisera, although the anti-C protein antisera (croC-141) was of low titer (Fig. 1C).

Engineered gene expression

Important to understanding flavivirus RNA replication will be determining the functional roles of the individual components involved. Toward this end, we exploited several gene expression systems for the production of individual YFV polypeptides. These expression systems included cell-free transcription-translation, mammalian cell transient expression, and recombinant baculovirus-infected insect (Sf9) cells. The first two systems both involve bacteriophage T7 promoter-initiated transcription (either *in vitro* or *in vivo*). We have engineered a number of the YFV protein coding regions into T7 promoter-containing plasmids (Fig. 2). Each of these transcription constructs was evaluated for protein expression in a transfection assay in BHK cells infected with a vaccinia virus expressing the T7 RNA polymerase (6). Cells transfected with the M construct produced an 8 kDa protein, immunoprecipitable with our anti-M-211 serum, that comigrated with the M protein produced in YFV-infected cells. Cells transfected with the prM plasmid synthesized an M antiserum-reactive 20 kDa protein, consistent with the size expected for the unglycosylated prM protein. Since the construct lacks the prM signal sequence, glycosylation was not expected. Cells receiving the CprM plasmid produced a specific 37 kDa polypeptide, the size expected if no proteolytic processing of the fusion protein occurred. Only a small amount of mature prM protein was detected. The presence of C protein was not tested. Cells transfected with the NS2B plasmid expressed the expected 15 kDa protein. Transient expression of the NS3 and NS5 constructs resulted in the ready identification of the expected 69 kDa and 98 kDa polypeptides, respectively. Finally, cells transfected with the NS3-5 plasmid produced very low levels of a high molecular weight polyprotein immunoprecipitable with antiserum to both the NS3 protein and NS5.

Five recombinant baculovirus transfer vectors possessing various YFV protein coding regions were constructed (Fig. 3). Four of these vectors were used to generate recombinant viruses. Analyses of YFV protein expression in recombinant virus-infected Sf9 cells showed bacM, bacNS3, and bacNS5 all produced the expected polypeptides, each co-migrating in an SDS polyacrylamide gel with their authentic YFV protein counterparts. However, bacNS2B-infected cells failed to produce an identifiable product.

RNA Replication Bioassay Development

To investigate viral RNA synthesis, we established methods for extraction of intact YFV RNA from virus-infected cells and for analysis of the various forms of viral RNA. Employing denaturing formaldehyde-agarose and high resolution urea-agarose gel

electrophoresis, differential LiCl solubility, and RNase sensitivity, we were able to readily resolve, distinguish, and identify viral genomic, replicative intermediate (RI), and replicative form (RF) RNAs.

Infected cell-derived RDRP assay.

A major effort was expended toward development of a cell-free viral RNA transcription assay derived from YFV-infected cells. We established a specific, reproducible, and quantifiable assay involving a subcellular fraction possessing viral RNA-dependent RNA polymerase (RDRP) activity. While similar crude RDRP activities have been developed for other flaviviruses, our work represents the first description of such an activity for YFV. Furthermore, this YFV RDRP activity was extensively characterized.

Methods employed to generate this active fraction were partially modeled after approaches used by others in different viral systems (7-12). Preparation of this fraction involved the mechanical disruption of YFV-infected BHK-21 cells in hypotonic buffer containing Triton X-100, followed by low speed centrifugation to remove large cell debris. From this initial supernatant fraction, a particulate fraction (P20) was isolated by centrifugation at 20,000 x g. The P20 fraction contained nearly all of the viral RDRP activity. This particulate material was then solubilized in deoxycholate (DOC) without loss of activity. Addition of glycerol to the DOC-solublized P-20 fraction allowed storage of aliquoted material at -70°C for several weeks with no apparent loss of RDRP activity.

Measurement of RDRP activity in this preparation involved standard reaction conditions including buffer, divalent cation, the four ribonucleoside triphosphates (NTPs), one of which is radiolabeled (α^{32}P-GTP), RNase inhibitor (RNasin), and actinomycin D. The RNA synthesizing activity of the P20 preparations was absolutely dependent on the addition of a divalent cation, with the optimal concentration of $MgCl_2$ being approximately 2.5 mM. Comparable activity was obtained at 0.5 mM $MnCl_2$. However, the activity was rather insensitive to variations in the nature and concentration of salt in the reaction mixture. Kinetic analyses showed viral RNA synthesis in this system was relatively short-lived, being essentially complete by 30 min. of incubation. Replenishing the reaction with NTPs after 30 minutes did not stimulate additional RNA synthesis.

Under standard reaction conditions in the presence of α^{32}P-GTP, the resultant radiolabeled product was analyzed by gel electrophoresis. Early in the reaction, radiolabel was incorporated exclusively into partially double stranded RI RNA. As the reaction progressed, this material was converted to completely double stranded RF RNA. Under no conditions were we able to identify single strand viral RNA. However, in other work, we found that the P20 extract contains an RNase activity as detected by the degradation of exogenously added RNA. All data from both the reaction product and kinetic analyses suggested the RDRP activity under study did not have the capacity to initiate

new RNA strand synthesis, but rather was capable only of completing RNA chains previously initiated *in vivo*.

Further purification of the RDRP activity present in the P20 fraction was accomplished by extraction with high salt followed by solublization with DOC. The resultant cleared fraction, designated CP20/NaCl, was enrich five-fold with respect to the RDRP activity. This purification resulted in the complete removal of the RNase activity mentioned above. However, the characteristics of the reaction and of the reaction products produced by the P20 and further-enriched CP20/NaCl fractions were essentially identical.

Analyses of the constituents of the RDRP-active P20 and CP20/NaCl cell fractions revealed both contained YFV-specific RF and single strand genomic RNA; very little RI RNA could be detected in either fraction. Using specific antiserum reagents, the viral proteins represented in the active fractions was analyzed. Both the P20 and CP20/NaCl fractions contained the full complement of viral polypeptides. However, the levels of the various proteins found in the CP20/NaCl fraction were greatly reduced relative to those in the P20 fraction. CP20/NaCl contained approximately 10-fold lower levels of all viral proteins except NS1. The level of NS1 in CP20/NaCl was about half that found in P20.

Rate-zonal centrifugation of the CP20/NaCl fraction on a glycerol gradient revealed a sedimentation coefficient for the active RDRP complex of approximately 21-24S. Analysis of the protein distribution within the gradient showed that greater than 90% of the protein sedimented as free protein near the top of the gradient (4-7S). Thus, the vast majority of the viral protein present in the CP20/NaCl fraction appears not to be associated with the RDRP activity. However, within the fractions of the glycerol gradient containing the RDRP activity, the full complement of YFV polypeptides could still be detected.

Attempts to identify the viral proteins involved in the RDRP activity in the glycerol gradient fractions using our panel of antiserum reagents were unsuccessful. None of the protein-specific antisera were able to precipitate the activity. Only the polyvalent YFV hyperimmune antiserum was capable of reacting with the active RDRP complex. Apparently, the epitopes recognized by the monospecific antibody reagents are inaccessible on the RDRP complex.

Thus, identification of the specific viral polypeptide(s) involved in, or responsible for, the observed RDRP activity remains to be determined. The difficulty in demonstrating unequivocally the specific proteins involved is further emphasized by the apparently extensive protein-protein associations observed among the viral polypeptides. Using our monospecific antiserum reagents, we have observed complex patterns of co-immuno-precipitation among the viral proteins.

Other enzymatic activities in RDRP-active fractions.

The CP20/NaCl fraction was assayed for other enzymatic activities suspected to be associated with viral RNA replication. Assays were established to measure guanine methyltransferase, RNA helicase, and ATPase activities. The CP20/NaCl preparation was shown to possess a poly(A)-independent ATPase activity not found in a similar cell fraction prepared form uninfected cells. However, this activity did not co-sediment with the RDRP activity in glycerol gradients. The nature of this ATPase activity was not elucidated. The CP20/NaCl fraction showed no guanine methyltransferase or RNA helicase activity under the assay conditions employed.

Reconstituted component replication assays.

Efforts toward purification of YFV proteins from YFV-infected cells and from recombinant baculovirus-infected cells were undertaken with the eventual goal being to reconstitute various replication-specific activities.

Attempts to purify the M protein from recombinant bacM-infected cells were initiated. The bacM protein was solubilized from disrupted cells by DOC, and remained soluble after removal of the detergent by dialysis. Conditions for ammonium sulfate precipitation and enrichment of the bacM protein were established. However, further efforts toward its purification were stopped upon notification of the research contract termination.

We found the vast majority of the NS3 protein in both authentic virus-infected and in bacNS3-infected cells to be present in the particulate fraction of hypotonically disrupted cells. Attempts to extract the NS3 protein with either a variety of detergents or high salt were unsuccessful. Use of urea and guanidine-HCl did result in release of varying levels of NS3 protein from the insoluble material. However, subsequent attempts to purify the solublized protein, employing a wide variety of fractionation procedures, failed to result in significant NS3 protein enrichment.

The majority (but not all) of NS5 protein was also found in the particulate fraction of both YFV- and bacNS5-infected cells. However, the protein from both sources was readily extracted and solubilized with high salt. These salt extracts were subjected to DEAE Sephacryl chromatography. Both NS5 proteins eluted similarly with a linear salt gradient, and the NS5 protein-containing fractions were then applied to a poly(U)-Sepharose column. Both NS5 proteins eluting from this column were estimated to be >90% pure.

In an attempt tc identify and associate any of the engineered proteins and purified protein with enzymatic activities, we developed several assays.

A poly(U) polymerase assay was developed to measure the incorporation of ^{32}P-UTP into TCA precipitable material using a poly(rA)·p(dT)$_{10}$ template-primer. Whereas purified recombinant poliovirus RDRP catalyzed a high level of poly(U) synthesis from

this template-primer, no activity was detected in highly purified preparations of recombinant baculovirus-produced or authentic YFV NS5. Moreover, we were unable to demonstrate RDRP activity using this template-primer in crude lysates of Sf9 cells infected with either bacNS3 or bacNS5, or in cells co-infected with bacNS3 and bacNS5. Finally, we were unable to detect poly(U) polymerase activity in YFV-infected SW-13 whole cell lysates.

The crude baculovirus NS3 protein preparation and the purified authentic and baculovirus recombinant NS5 proteins were assayed for ATPase activity. We found the crude NS3 preparation possessed activity. This ATPase activity was stimulated almost 2-fold by the addition of poly(A) to the reaction mixture, while the addition of poly(C) or poly(U) had no affect ATP hydrolysis. While stimulation of ATP hydrolysis by poly(A) is consistent with findings reported for RNA-dependent helicases from other sources, considerable work remains to determine if the observed activity is attributable to the NS3 polypeptide. No ATPase activity was detected in the purified NS5 protein preparations.

An RNA helicase assay was established. Utilizing a number of transcription vectors, pairs of RNA transcripts were produced in such a manner that, when annealed, formed partially double-stranded RNAs. The longer RNA of a pair was prepared unlabeled, while the shorter RNA was synthesized in the presence of [α^{32}P]-GTP. Helicase activity was measured by incubating these substrates with protein fractions, and then subjecting the reaction mixture to electrophoresis on a SDS-containing polyacrylamide gel. The presence of helicase activity was revealed by the release of the radiolabeled short RNA from the hybrid.

An RNA helicase activity was detected in the purified authentic NS5 protein preparation, but not in the recombinant baculovirus material. The activity was dependent on divalent cations and also on the addition of NTPs at millimolar concentrations. There was no preference for any of the NTPs. The activity was stimulated several-fold by addition to the reaction mixture of single-stranded RNA. The stimulating RNA could be homopolymer [poly(A)], heteropolymer [poly(ACU)], or specific RNA transcripts synthesized *in vitro*. Unfortunately, the nature of the observed helicase could be cellular. Material prepared from uninfected BHK cells similarly fractionated as for the purification of the NS5 protein also possessed an RNA helicase activity.

Both purified NS5 proteins were assayed for guanine methyltransferase activity. Using either GTP or GpppG as methyl acceptors, and ^{3}H-S-adenosyl methionine as methyl donor, we were unable to detect activity in either preparation.

Inhibitor Design and Development

Due to the abbreviated contract term, our entire effort in the area of RNA replication inhibitor candidates was compromised and was restricted to a minimal consideration of oligopeptides

only. In our limited efforts, peptide sequences derived from two regions within the NS5 protein, the "SG" and "GDD" motifs, were selected for study based on their conserved nature among RNA replicases (13).

Evaluation of candidate inhibitors.

With the infected cell-derived soluble RDRP assay being established as reproducible and quantifiable, we initiated efforts toward evaluation of the effects on this activity of the addition of several peptides. A series of hexamers, based on conserved sequence motifs in the NS5 protein, were designed, synthesized, and tested. Synthetic peptides representing these conserved sequences (mimetic peptides) were made, as were peptides predicted to be "complementary" to them. The basis for design of complementary peptides stems from the theory of hydropathic anti-complementarity put forth by Blalock and Smith (14).

At final peptide concentrations in the reaction mixture of 50 and 250uM, none of the "GDD" mimetic peptides showed inhibitory activity. However, when tested at 1250uM, the GDD compound MedImmune (M) 96-10 exhibited significant inhibitory effect on RDRP activity (42% inhibition), while at this same concentration, GDD complementary hexamers M96-12, -13 and -14, and an unrelated hexamer (B3/2-7) had only minor effects on activity (0-15% inhibition). The "SG" hexamer M96-15 had only a slight inhibitory effect, even at 1250 uM. However, three SG complementary hexamers (M96-16, -17 and -18) all exhibited significant RDRP inhibition at the high peptide concentration; M96-16 and M96-17 being most potent (42% inhibition). Analysis of the reaction products synthesized in the absence or presence of 1250uM M96-10 or M96-17 on a denaturing formaldehyde-agarose gel revealed a clear decrease in the amount of radiolabeled YFV-specific 11 kb RNA made in the presence of each of these peptides.

Since inhibition of RDRP activity was observed only at very high peptide concentrations (mM), in order to determine to significance of these observations, we synthesized additional peptides and evaluated them in the cell-derived RDRP system. Two peptides representing the M96-17 sequence randomized (M96-21 and -22) exhibited no inhibitory activity. Evaluation of peptides in which single alanine substitutions were made for several of the residues in the M96-17 peptide (compounds M96-33 to M96-37), in every case resulted in loss of inhibitory activity. Of two randomized peptides of M96-10, one (M96-19) showed no inhibition, while the other (M96-20) inhibited the reaction to a similar extent as did M96-10. Deleting either terminal amino acid (M96-30 and -31), or both terminal amino acids (M96-23), in compound M96-10 abrogated inhibitory activity.

Two new compounds designed to target and inhibit activities associated with the NS3 protein were synthesized, but time did not permit their functional evaluation.

While our initial observations and results are intriguing and potentially very exciting, considerable additional work is required to validate specific inhibitory activities.

Molecular modeling studies.

Computational analyses involving the application of dynamics and minimization techniques were initiated to investigate the conformation of these peptides. We employed the program "Discover" (Biosym Technologies) using Silicon Graphics (SGI) computers at USAMRIID in collaboration with Maj. Dallas Hack and the Cray X-MP computer at NCI-FCRF. An extensive dynamics, annealing, and minimization analysis of one cyclic hexamer (compound M96-11) indicated there was a limit to the unique conformational space it can occupy. Data generated by this procedure were analyzed by three distinct methods so as to classify each conformer into a conformational group or family. The first structural characterization method employed the program RMSfit (Biosym Technologies). RMSfit groups peptides having within 1 angstrom or less the same average root mean squared distance between all backbone atoms of the peptide. The second characterization method used a computer program called Conf (written by D.C. Feller and D.C. Hack). Conf assigns each residue of a peptide a conformational letter code according to the residue's phi and psi dihedral angles. Conformers having the same letter codes for all residues are grouped into a family. The third characterization method considers predicted secondary structure trends of each conformer (α-helix, β-sheet, β-turn).

These computational analyses were to form an initial conformational database for future inhibitor design.

Summary and Conclusions

We believe our efforts over the 21 month tenure of this contract have been productive and fruitful. We established our study system and assembled all the reagents necessary for future detailed studies of RNA replication. Several YFV protein coding regions were engineered into various surrogate expression systems, and the recombinant proteins thereby produced would have served as important sources of material for functional biochemical studies. Our progress in the identification and characterization of an RDRP activity derived from virus-infected cells was considerable and resulted in the establishment of an assay that was specific, reproducible, and readily quantifiable. The availability of this assay allowed us to initiate studies involving the evaluation of compounds for RNA replication inhibitory activity. Our data suggest we discovered peptide compounds with inhibitory activity. However, due to the premature termination of the contract, efforts to validate the specificity and selectivity of these lead compounds, and to investigate their possible mechanisms of action were not permitted.

We are very disappointed by the decision to terminate this contract. We were confident our molecular and biochemical approaches to drug discovery and development for flaviviruses and related RNA virus pathogens was unique, promising, and highly relevant to military medicine. It was our objective to apply

modern strategies in antiviral drug design and discovery, currently being implemented successfully for retroviruses and several DNA viruses, to several groups of RNA viruses which are of particular military medical interest or of biological warfare defense concern. We feel the decision to terminate this contract deprives the military of an outstanding opportunity to exploit the current biotechnology industry revolution in rational antiviral drug development.

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Figure 1

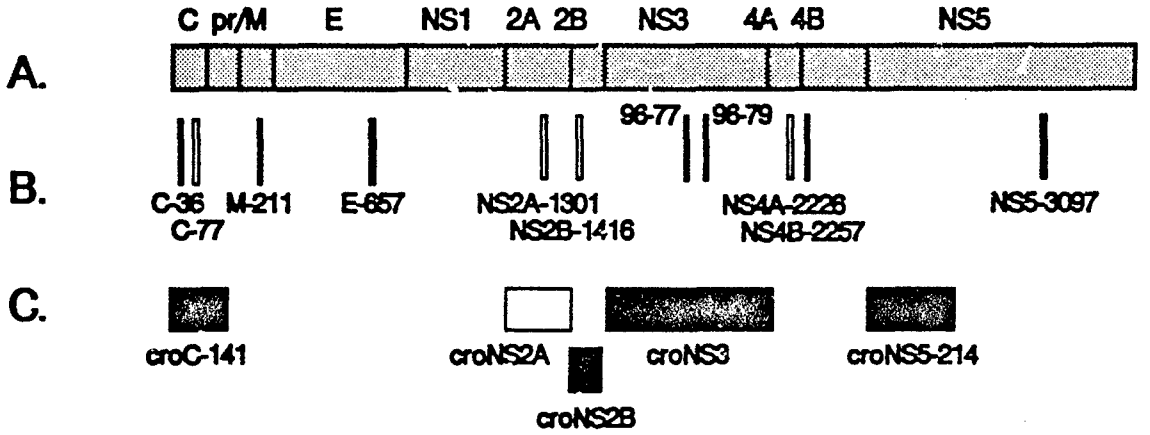

FIGURE 1. Sequence-specific antiserum reagents to YFV proteins. A. Schematic representation of the YFV ORF, indicating the coding boundaries for each of the viral proteins. B. Synthetic peptides (13-22mers) prepared and used as immunogens. The number following the protein designation indicates the amino acid position witnin the ORF of the first residue of the respective peptide. Peptide 96-77 begins at residue 1800 and peptide 96-79 at residue 1940, both within the NS3-coding region. The open bars indicate antisera elicited to the immunogen failed to recognize the respective YFV protein. Solid bars indicate useful antisera were generated. C. Regions of the YFV ORF engineered into *E. coli* expression vectors. In all but one case (croNS2A), fusion proteins were produced in bacteria harbouring these vectors. Immunization with these proteins resulted in the generation of useful antisera reactive with the authentic YFV proteins they each represented.

Figure 2

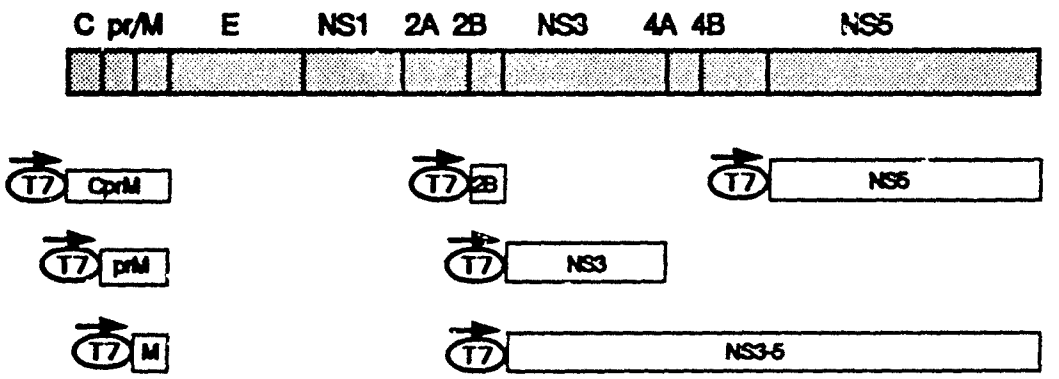

FIGURE 2. YFV-T7 transcription plasmids. The indicated protein coding regions of the YFV ORF were engineered into transcription plasmids downstream of the bacteriophage T7 promoter sequence.

Figure 3

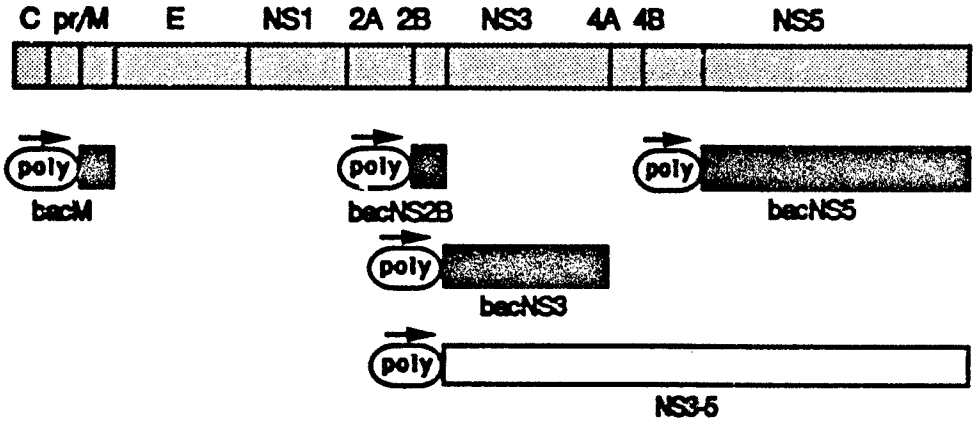

FIGURE 3. YFV-Recombinant baculoviruses. The indicated protein coding regions of the YFV ORF were engineered into baculovirus transfer vectors (pVL1392) downstream of the polyhedrin promoter sequence. The shaded constructs were then used to generate recombinant viruses.